

Arginase I, Polyamine, and Prostaglandin E₂ Pathways Suppress the Inflammatory Response and Contribute to Diffuse Cutaneous Leishmaniasis

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Diffuse cutaneous leishmaniasis (DCL) is a rare clinical manifestation of tegumentary leishmaniasis. The molecular mechanisms underlying DCL pathogenesis remain unclear, and there is no efficient treatment available. This study investigated the systemic and in situ expression of the inflammatory response that might contribute to suppression in DCL. The plasma levels of arginase I, ornithine decarboxylase (ODC), transforming growth factor β (TGF- β), and prostaglandin E₂ (PGE₂) were higher in patients with DCL, compared with patients with localized cutaneous leishmaniasis (LCL) or with controls from an area of endemicity. In situ transcriptomic analyses reinforced the association between arginase I expression and enzymes involved in prostaglandin and polyamine synthesis. Immunohistochemistry confirmed that arginase I, ODC, and cyclooxygenase2 expression was higher in lesion biopsy specimens from patients with DCL than in those from patients with LCL. Inhibition of arginase I or ODC abrogates *L. amazonensis* replication in infected human macrophages. Our data implicate arginase I, ODC, PGE₂, and TGF- β in the failure to mount an efficient immune response and suggest perspectives in the development of new strategies for therapeutic intervention for patients with DCL.

Keywords. *Leishmania amazonensis*; diffuse cutaneous leishmaniasis; arginase I; ornithine decarboxylase; prostaglandin E₂; TGF- β .

Cutaneous leishmaniasis exhibits a wide spectrum of clinical manifestations varying from self-healing localized cutaneous leishmaniasis (LCL) with a moderate cell-mediated immune response to diffuse cutaneous leishmaniasis (DCL) [1]. DCL is distinct from dissemi-

nated cutaneous leishmaniasis [2] and is characterized by the presence of several nonulcerated nodular skin lesions, the predominance of highly parasitized macrophages in the lesions, an absent or modest in vitro antileishmanial antigen cellular immune response, a negative delayed-type hypersensitivity (DTH) response, and resistance to antiparasite therapy [3]. The molecular mechanisms underlying DCL pathogenesis remain unclear, and there is no efficient treatment available.

In patients with DCL, antiinflammatory cytokines are abundant in lesions and in restimulated peripheral blood mononuclear cells (PBMCs), whereas proinflammatory cytokines and chemokines are absent or present at low levels [1]. However, the mechanisms responsible for this imbalance are not yet understood.

The arginase I pathway is emerging as a critical mechanism of immune regulation in *Leishmania* infection [4]

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and is highly expressed in lesions from patients with LCL [5]. This enzyme metabolizes L-arginine into urea and L-ornithine, the substrate used for ornithine decarboxylase (ODC) to produce polyamines that are crucial for parasite replication. Macrophages infected with *Leishmania major* or *Leishmania infantum* and treated with LOHA (L-hydroxyl arginine), an arginase inhibitor, exhibit significant decrease in parasite load [6]. Moreover, DFMO, a potent ODC inhibitor, effectively inhibits *Leishmania donovani* promastigote growth in culture [7].

Leishmania amazonensis, the sole agent implicated in DCL in Brazil [8], increases arginase I, transforming growth factor β (TGF- β), and prostaglandin E₂ (PGE₂) expression, contributing to intra-macrophage parasite proliferation [9–11]. Nevertheless, the relevance of these mediators in DCL pathogenesis remains unknown. In the present study, we investigated the systemic levels and in situ expression of inflammatory mediators in samples from patients with DCL, patients with LCL, and healthy controls, who were identified among family members of patients with DCL. High levels of arginase I, ODC, PGE₂, and TGF- β were observed in plasma specimens from patients with DCL. In addition, in situ transcriptomic analyses reinforced the association between arginase I expression and the enzymes involved in the pathways of prostaglandin and polyamine biosynthesis at the messenger RNA (mRNA) level. Moreover, arginase and ODC inhibitors prevented parasite replication and modulated inflammatory mediators in human monocyte-derived macrophages infected with *L. amazonensis*. Our study highlights that arginase I, ODC, and COX-2 biosynthetic enzymes can be used as potential drug targets for *Leishmania* infection.

MATERIALS AND METHODS

Ethics Statement

Written informed consent was obtained from all participants or legal guardians, and all data analyzed were anonymized. The project was approved by the institutional review board of Centro de Pesquisas Gonçalo Moniz, FIOCRUZ–BA (license number 136/2007), and comply with the guidelines of the Declaration of Helsinki.

Patient Characteristics

All patients with DCL (n = 12) were followed by one of the authors (J. M. C.), and their characteristics have been reported previously [3]. Their diagnoses were established as described elsewhere [12]. Patients with DCL exhibited a chronic evolution of the disease with several remissions, multiple nodular and highly parasitized lesions throughout the skin, and a negative DTH response. Patients with LCL (n = 29) with a single or few ulcerated lesions present for up to 2 months and a positive DTH response were followed by one of the authors (A. B.) [13]. The clinical and epidemiological data from patients with DCL and those with LCL are summarized in Table 1. Controls were

Table 1. Epidemiological and Clinical Parameters for Patients With Diffuse Cutaneous Leishmaniasis (DCL) and Those With Localized Cutaneous Leishmaniasis (LCL)

Characteristic	LCL Group (n = 29)	DCL Group (n = 12)	P value
Sex, no.			.09
Male	19	7	
Female	10	5	
Age, y			.06
Mean \pm SD	34 \pm 15	17 \pm 23	
Range	13–65	4–41	
Active lesions, no.			.001
Mean	1	138	
Range	1–9	22–500	
DTH positivity, %	100	0	.0001
Disease duration, mo			.0001
Mean	3	142	
Range	1–6	36–276	

Abbreviations: DTH, delayed hypersensitivity skin-test response; SD, standard deviation.

identified among 49 family members of the patients with DCL and underwent careful physical examinations, exhibiting no cutaneous lesions or prior CL history and a negative DTH response. Individual blood samples were collected after examination. Skin biopsy specimens (3 or 4 mm) were obtained from 4 patients with DCL and 7 patients with LCL, embedded in a cryopreservation resin, snap frozen, and stored in liquid nitrogen. We did not have lesion biopsy specimens available from all patients during the period of this study, and since the procedure is too invasive not all patients have allowed collection of a new lesion biopsy specimen.

Inflammatory Mediator Measurements

Plasma levels of arginase I (Hycult Biotech, Uden, the Netherlands), ODC (Wuhan EIAAB Science, Wuhan, China) and TGF- β (R&D Systems, Minneapolis, Minnesota) were measured using enzyme-linked immunosorbent assay ELISA according to the manufacturer's instructions. The total TGF- β level was measured in plasma after acidification, according to the manufacturer's instructions. The plasma levels of interleukin 10 (IL-10), interleukin 12 (IL-12), interferon γ (IFN- γ), monocyte chemotactic protein 1 (MCP-1), CXCL-10, and tumor necrosis factor α (TNF- α) were measured using a Cytometric Bead Array (CBA) Human Inflammatory kit (BD Biosciences Pharmingen, San Diego, California) according to the manufacturer's protocol. The flow cytometry assay was performed and analyzed by a single operator. PGE₂ production was measured using a specific enzyme immunoassay (the PGE₂ EIA kit, Cayman Chemical, Ann Arbor, Michigan) according to the manufacturer's instructions. TGF- β , PGE₂, TNF- α , and IL-12 expression was measured in supernatants by the same protocols.

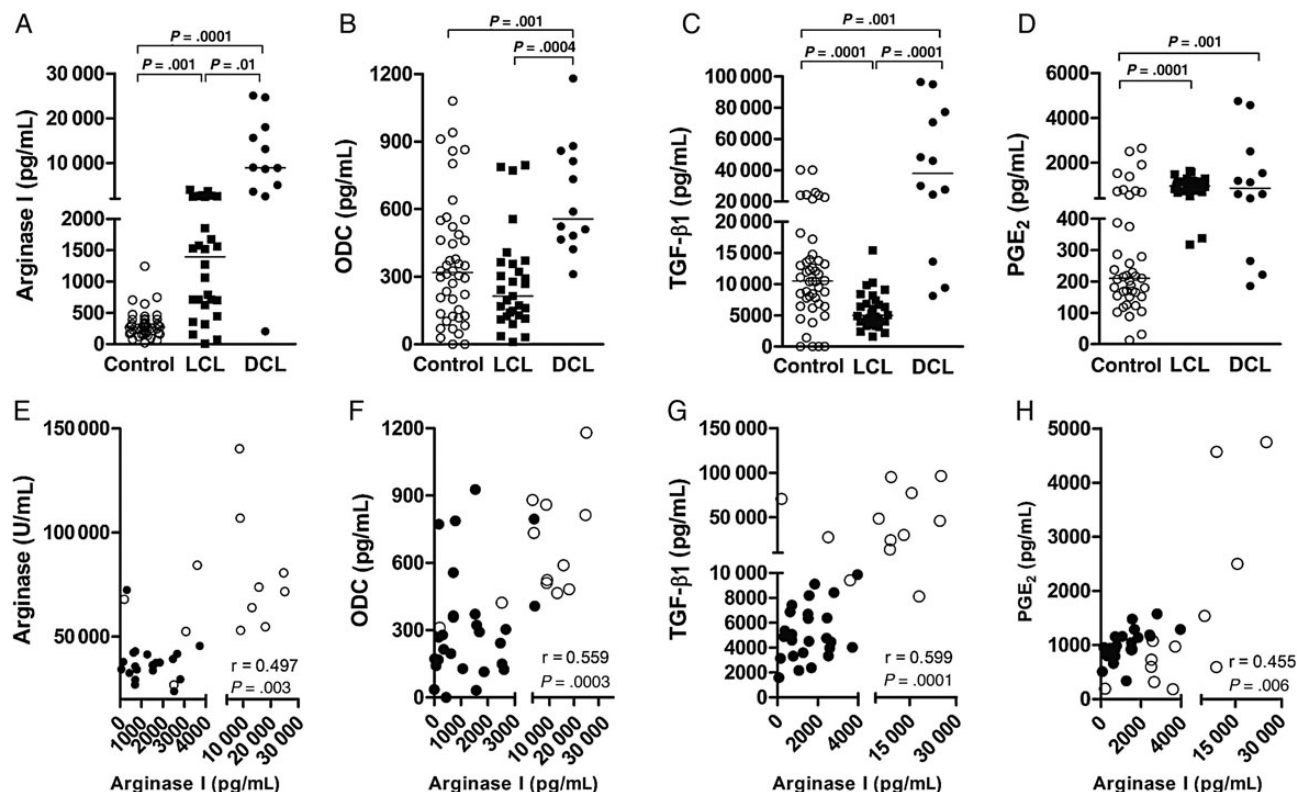


Figure 1. Arginase I, ornithine decarboxylase (ODC), and antiinflammatory mediators in plasma specimens from patients with diffuse cutaneous leishmaniasis (DCL) and those with localized cutaneous leishmaniasis (LCL). Plasma protein levels of arginase I (A), ODC (B), transforming growth factor β (TGF- β ; C), and prostaglandin E_2 (PGE $_2$; D) were measured by an enzyme-linked immunosorbent assay in healthy controls ($n = 35$), patients with active DCL ($n = 12$), and patients with localized cutaneous leishmaniasis (LCL; $n = 30$). Correlations between arginase I and arginase activity (E), ODC (F), TGF- β (G), and PGE $_2$ (H) were calculated. Each point represents a different donor, and each bar represents the median. Open circles represent patients with DCL, and closed circles represent patients with LCL. Differences between groups were tested by the Kruskal–Wallis test with the Dunn multiple comparisons post hoc test. The Spearman test was used to verify the significance of correlations between parameters. Data are representative of at least 2 independent assays performed in duplicate for each patient or control.

Measurement of Arginase Enzymatic Activity

Arginase activity was measured by a colorimetric assay for the detection of urea in plasma samples, as previously described [14].

Immunohistochemistry

Immunohistochemistry was performed as previously reported by us [15], using primary antibodies against human arginase I (2 μ g/mL; 1:100; Santa Cruz Biotechnology, Dallas, Texas), ODC (4 μ g/mL; 1:100; Cayman Chemical, Ann Arbor, Michigan), and COX-2 (2 μ g/mL; 1:50; Cayman Chemical, Ann Arbor, Michigan). Digital images were obtained from fields of 400 \times original magnification and captured using a Nikon E600 microscope and an Olympus Q-Color 1 digital camera with the Image-Pro Plus program.

nCounter Analysis

Total RNA was extracted from lesion biopsy specimens from 4 patients with DCL and 7 patients with LCL, using TRIzol, according to the manufacturer's protocol, with an additional purification step performed using RNeasy columns (Qiagen

Benelux, Venlo, the Netherlands). nCounter (NanoString Technologies, Seattle, Washington) analysis was performed at the VIB MicroArray Facility (Leuven, Belgium) based on direct molecular bar coding of target RNA transcripts and digital detection [16]. Through the use of color-coded probe pairs and direct hybridization and without the use of reverse transcriptase or amplification, the following host-specific cellular genes were quantified: *ARG1*, which encodes arginase I; *ODC*, which encodes ornithine decarboxylase; *EP1*, which encodes prostaglandin E receptor 1; *EP2*, which encodes prostaglandin E receptor 2; *EP3*, which encodes prostaglandin E receptor 3; *EP4*, which encodes prostaglandin E receptor 4; *PLA2G4A*, which encodes phospholipase A2, group IVA (cytosolic, calcium-dependent); *PTGS1*, which encodes prostaglandin-endoperoxide synthase 1; *PTGS2*, which encodes prostaglandin-endoperoxide synthase 2; *PTGES*, which encodes prostaglandin E synthase; *SMS*, which encodes spermine synthase; and *SRM*, which encodes spermidine synthase. The following housekeeping genes were also quantified for normalization at the femtomolar range: *GUSB*,

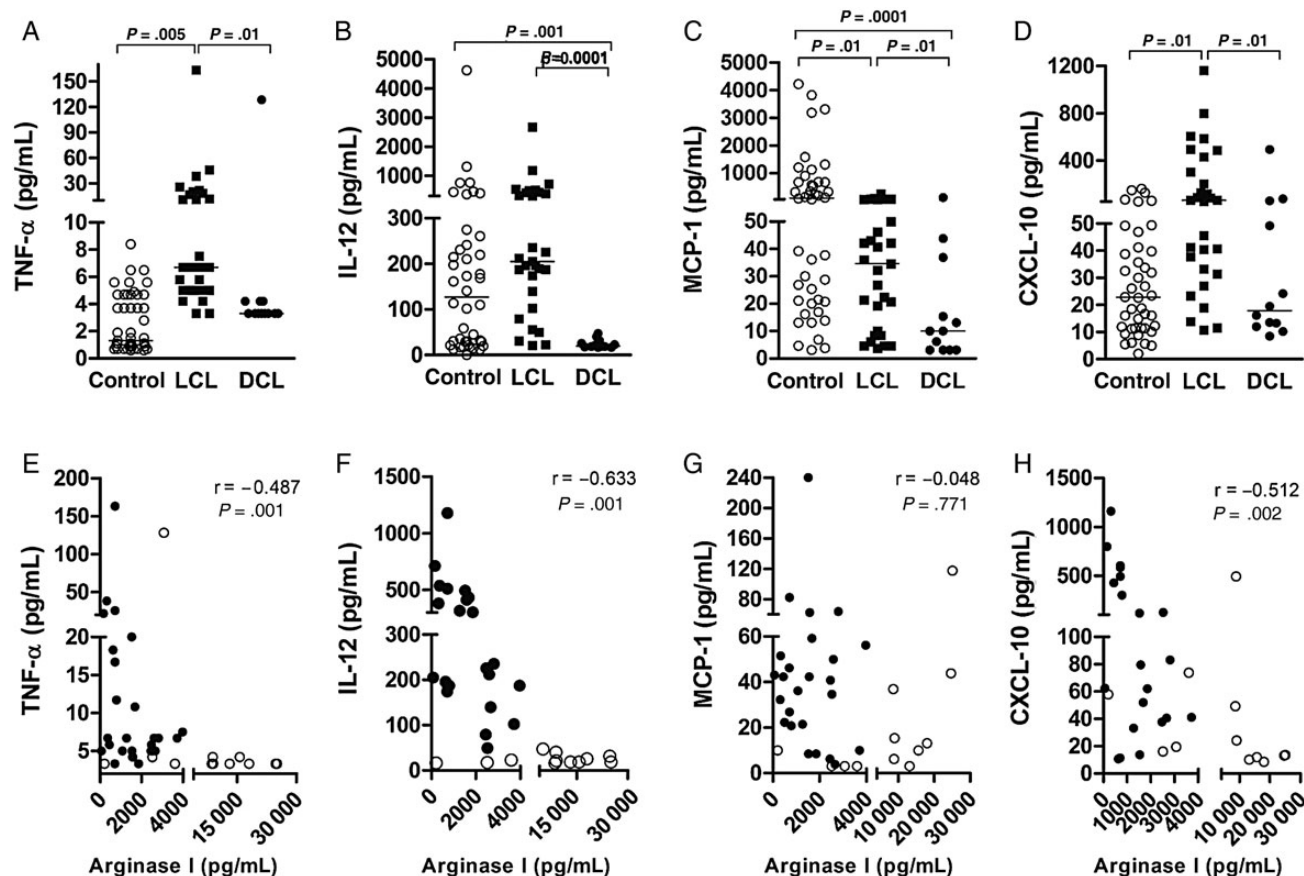


Figure 2. Proinflammatory cytokines and chemokines in plasma specimens from patients with diffuse cutaneous leishmaniasis (DCL) and those with localized cutaneous leishmaniasis (LCL). Plasma protein levels of tumor necrosis factor α (TNF- α ; A), interleukin 12 (IL-12; B), monocyte chemotactic protein 1 (MCP-1; C), and CXCL-10 (D) were measured by the Cytometric Bead Array kit in healthy controls ($n = 35$), patients with active DCL ($n = 12$), and patients with LCL ($n = 30$). Correlations between arginase I and TNF- α (E), IL-12 (F), MCP-1 (G), and CXCL-10 (H) levels were measured. Each point represents a different individual, and each bar represents the median. Open circles represent patients with DCL, and closed circles represent LCL patients. Differences between groups were tested by the Kruskal–Wallis test with the Dunn multiple comparisons post hoc test. The Spearman test was used to verify the significance of correlations between parameters. Data are representative from at least 2 independent assays performed in duplicate for each patient or control.

which encodes β -glucuronidase; *G6PD*, which encodes glucose-6-phosphate dehydrogenase; *GAPDH*, which encodes glyceraldehyde-3-phosphate dehydrogenase; *HPRT1*, which encodes hypoxanthine phosphoribosyltransferase; and *CD45*, which encodes the pan-leukocyte marker CD45.

Cell Culture

PBMCs and monocytes were isolated and cultured as previously described [17]. The promastigotes used in these experiments came from an *L. amazonensis* strain (MHOM/BR/87/BA336) isolated from a patient with DCL. The methods of parasite culture and macrophage infection have been previously reported [18]. Macrophages were infected with early stationary phase *L. amazonensis* at a parasite to cell ratio of 6:1. After 4 hours of incubation at 34°C, free parasites were removed by extensive washing with phosphate-buffered saline, and 10 μ g/mL nor-NOHA or 0.5

mM DFMO (arginase and ODC inhibitors) was added to the cultures (both from Sigma-Aldrich, St. Louis, Missouri). The intracellular parasite load was estimated at 24 and 72 hours after infection by light microscopy and the production of viable promastigotes in Schneider medium, as described previously [18].

Statistical Analysis

For ordinal variables, differences between groups were calculated using the nonparametric Kruskal–Wallis test with the 2-tailed Dunn multiple comparisons post hoc test and the Mann–Whitney unpaired *t* test for 2-group comparisons. The χ^2 test was used to compare differences between categorical variables. The Spearman test was used to verify the significance of correlations between arginase I and the plasma levels of arginase activity, ODC, TGF- β , PGE₂, TNF- α , IL-12, MCP-1, IFN- γ , and CXCL-10 (nonparametric data). For transcriptomic

(nCounter) analysis, in situ mRNA levels were normalized to CD45 mRNA levels, followed by log transformation, allowing linear regression analysis of the interactions between inflammatory mediators, as previously described [19,20]. The values in the text represent medians \pm standard deviations. Differences were considered statistically significant at P values of $\leq .05$. Data were analyzed using GraphPad Prism 5.0 (GraphPad Software).

RESULTS

Inflammatory Imbalance Mediators in Plasma Specimens From Patients With DCL

Plasma specimens from patients with DCL exhibited the highest arginase I levels, by at least 5-fold, compared with patients with LCL or controls (Figure 1A). Patients with DCL also exhibited higher arginase activity (69.850 ± 29.017 U/mL) than patients with LCL (36.602 ± 9.659 U/mL) and controls (28.971 ± 11.037 U/mL). L-Ornithine, the arginase product, can be used by ODC, resulting in polyamine production [14]. ODC was augmented in DCL plasma when compared with the control and LCL groups (Figure 1B).

We next investigated the mediators known to modulate the L-arginine metabolic pathways. TGF- β displayed higher levels in plasma from patients with DCL than patients with LCL (Figure 1C). Similarly, PGE₂ levels were higher in patients with DCL than in controls, but no difference was detected between patients with DCL and patients with LCL (Figure 1D). Arginase I exhibited a positive correlation with antiinflammatory mediators, including arginase activity, ODC, TGF- β , and PGE₂ (Figure 1E–H). However, the proinflammatory cytokines and chemokines TNF- α (Figure 2A), IL-12 (Figure 2B), MCP-1 (Figure 2C), and CXCL-10 (Figure 2D) were reduced in plasma specimens from the DCL group, compared with levels in plasma from the LCL and control groups. Arginase I exhibited a negative correlation with TNF- α , IL-12, and CXCL-10 (Figure 2E–H) but not with MCP-1 (Figure 2G).

To determine whether arginase I, ODC, and COX-2 were also expressed in situ, we performed a comprehensive analysis of genes implicated in this inflammatory pathways. The mRNA levels of the selected genes were quantified and normalized to the mRNA level of the pan-leukocyte marker CD45 to correct varying levels of leukocyte infiltration between patient lesion biopsy specimens. Cells in lesion biopsy specimens from the DCL group exhibited significantly increased arginase I mRNA levels, compared with those from the LCL group (Table 2), corroborating the increased levels of this enzyme found in plasma specimens from patients with DCL. However, the ODC mRNA levels did not significantly differ between the DCL and LCL groups. The mRNA levels of IL-4 and IL-10 were significantly increased, although the TNF- α mRNA level was decreased in lesions from the DCL group in situ (Table 2). PGE₂ binds to 4 cellular receptors, of which EP2 mRNA was significantly overexpressed in

Table 2. Messenger RNA (mRNA) Levels of Inflammatory Mediators in Lesion Biopsy Specimens From Patients With Diffuse Cutaneous Leishmaniasis (DCL) and Those With Localized Cutaneous Leishmaniasis (LCL)

Mediator	LCL Group (n = 7)	DCL Group (n = 4)	P value
Arginase I	0.02	1.02	<.04
ODC	−0.63	−0.88	<.64
EP-2	−0.96	−0.53	<.006
PLA2G4A	−1.28	−1.02	<.09
Interleukin 4	−3.02	−1.72	<.02
Interleukin 10	−2.47	1.66	<.01
TNF- α	−0.35	−0.68	<.01

Values were obtained by nCounter digital quantification of mRNA levels and normalized to the CD45 mRNA level, followed by log transformation. Negative values thus reflect a ratio of mediator mRNA level to CD45 mRNA level of <1.

Abbreviations: EP-2, prostaglandin E receptor 2; ODC, ornithine decarboxylase; PLA2G4A, phospholipase A2, group IVA; TNF- α , tumor necrosis factor α .

lesion biopsy specimens from the DCL group, compared with those from the LCL group (Table 2). Present upstream of PGE₂, phospholipase A2 is the crucial enzyme for arachidonic acid production, and levels of this enzyme were significantly higher in DCL lesions, compared with LCL lesions (Table 2).

As shown in Figure 3, arginase I mRNA levels were positively correlated with PLA2G4A, PTGES, and PTGS2 mRNA levels (Figure 3A–C), all of which are enzymes involved in prostaglandin synthesis. Arginase I expression was also positively correlated with spermine synthase and spermidine synthase expression (Figure 3D and 3E), which are enzymes responsible for polyamine synthesis.

Arginase I, ODC, and COX-2 Expression in Lesion Biopsy Specimens From Patients With DCL

Arginase I staining was more widespread in lesion biopsy specimens from the DCL group (n = 3) than in those from the LCL group (n = 3), as shown in Figure 4. The expression of ODC and COX-2, which are involved in polyamine and PGE₂ synthesis, respectively, exhibited an increase in DCL lesions when compared with LCL lesions. No reactivity was detected using an isotype control antibody (data not shown).

Arginase and ODC Inhibition Control *L. amazonensis* Infection in Human Macrophages

Macrophages infected with *Leishmania* organisms exhibited $10.9 \times 10^4 \pm 2.1 \times 10^4$ viable parasites at 24 hours after infection. The groups treated with nor-NOHA or DFMO presented no difference in parasite load at the same time (Figure 5A). However, 72 hours after infection, the unstimulated cells group showed parasite proliferation ($6.2 \times 10^7 \pm 1.75 \times 10^7$), and the groups treated with nor-NOHA and DFMO exhibited $3.6 \times 10^4 \pm 6.05 \times 10^4$ and $1 \times 10^5 \pm 1.40 \times 10^4$ viable parasites, respectively,

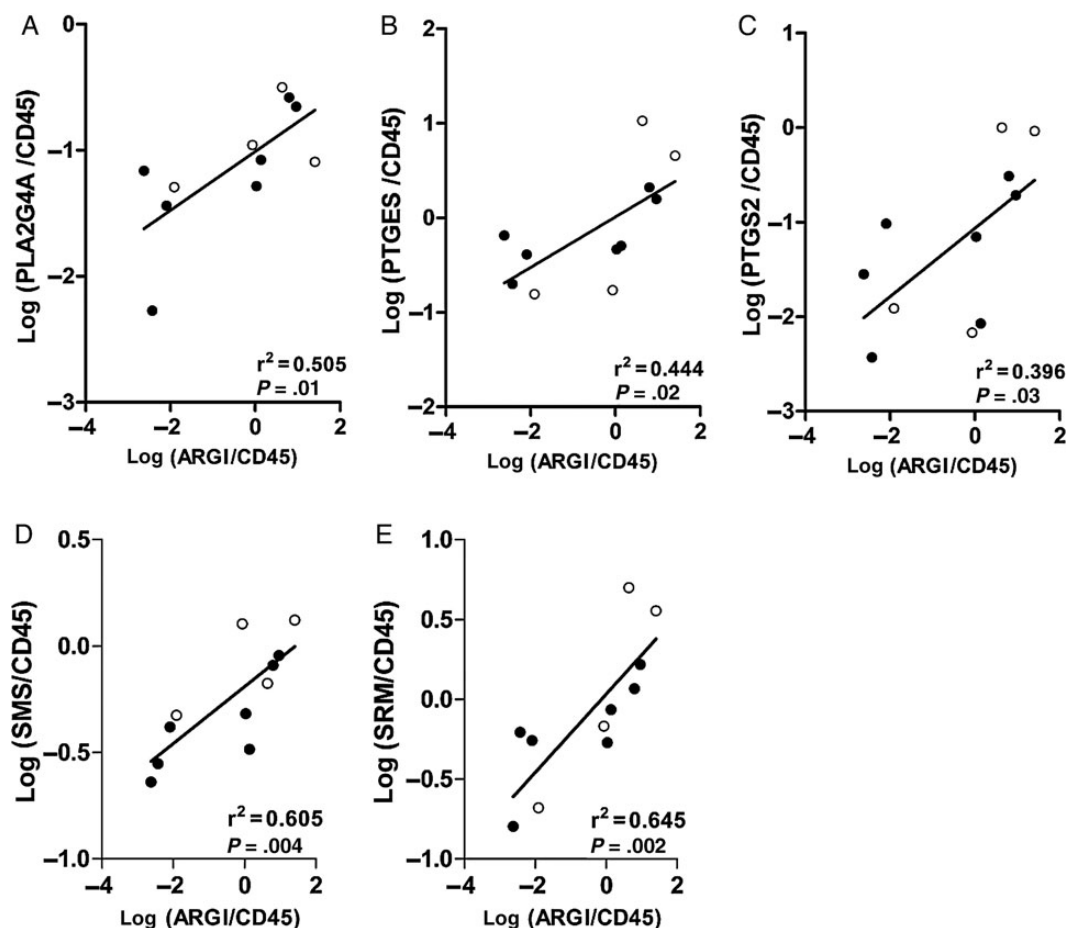


Figure 3. Arginase I in situ is related to polyamine and PG pathways in lesion biopsy specimens from patients with diffuse cutaneous leishmaniasis (DCL) and those with localized cutaneous leishmaniasis (LCL). Total RNA was extracted from lesion biopsy specimens for patients with LCL ($n = 7$) and those with DCL ($n = 4$), and the messenger RNA transcripts of host-specific cellular genes were quantified by nCounter (Nanostring), including that of a housekeeping gene encoding CD45, for normalization. Linear regression between ARG1 (which encodes arginase I) and PLA2G4A (which encodes phospholipase A2; (A), PTGES (which encodes prostaglandin E synthase; (B), PTGS2 (which encodes prostaglandin-endoperoxide synthase [known as cyclooxygenase 2]; (C), and the polyamine biosynthetic pathways for SMS (which encodes spermine synthase; (D) and SMR (which encodes spermidine synthase; (E). Open circles represent patients with DCL, and closed circles represent patients with LCL. The statistical significance of differences was determined by linear regression. The data were collected in triplicate for each donor.

which were both significantly lower than the level in unstimulated cells (Figure 5A). In addition, at 72 hours after infection, photomicrographs confirmed that cells treated with nor-NOHA and DFMO were able to control the parasite burden more efficiently than cells in the untreated group (Figure 5B).

Next, we measured essential mediators of *L. amazonensis* survival and proliferation in human macrophage culture supernatants at 24 hours. First, we observed that nor-NOHA or DFMO treatment decreased TGF- β (Figure 5C) and PGE₂ (Figure 5D) production by at least 50%, compared with the control group. Subsequently, we measured the levels of key cytokines that control *Leishmania* infection. nor-NOHA-treated cells exhibited a 3-fold increase in both TNF- α (Figure 5E) and IL-12 (Figure 5F) levels, whereas DFMO-treated cells did not exhibit altered cytokine levels, compared with untreated cells.

DISCUSSION

DCL is characterized by an inefficient parasite-specific cellular response and heavily parasitized macrophages. However, the immunopathogenic mechanisms underlying this disease remain unclear. The findings reported here lead us to propose a straightforward pathway by which arginase I, ODC, PGE₂, and TGF- β contribute to DCL lesions, thus resulting in a permissive microenvironment for *Leishmania* proliferation and progression to chronic disease.

Plasma from patients with DCL exhibited higher levels of arginase I expression and activity, compared with plasma from patients with LCL or controls. High levels of arginase activity in plasma and saliva have been reported in several other human pathologic conditions as a useful biological biomarker,

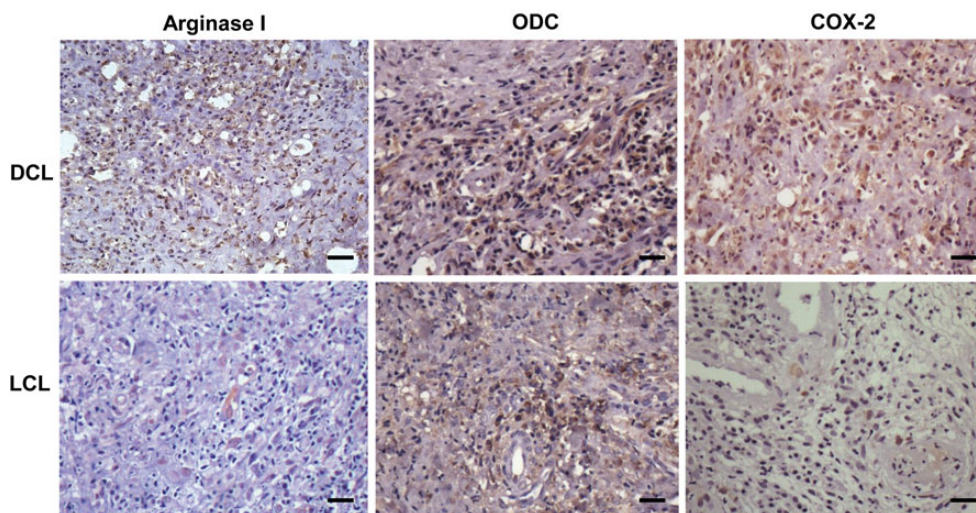


Figure 4. Arginase I, ornithine decarboxylase (ODC), and COX-2 expression in lesions biopsy specimens from patients with diffuse cutaneous leishmaniasis (DCL) and those with localized cutaneous leishmaniasis (LCL). Immunohistochemistry was performed on paraffin-embedded sections of lesion biopsy specimens from patients with DCL and those with LCL, using primary antibodies against arginase I (2 $\mu\text{g}/\text{mL}$; 1:100), ODC (4 $\mu\text{g}/\text{mL}$; 1:100), and COX-2 (2 $\mu\text{g}/\text{mL}$; 1:50). The labeling was revealed with diaminobenzidine (DAB), followed by counterstaining with Harris hematoxylin. Data are representative of at least 3 independent assays and were collected in duplicate for each patient. Digital images (400 \times original magnification) were captured using a Nikon E600 microscope and an Olympus Q-Color 1 digital camera with the Image Pro Plus program. Bars represent 10 μm .

in some cases representing an indicator of disease progression [21, 22], but the mechanism by which arginase I is released remains unclear. Recently, increased arginase I activity was demonstrated in situ in LCL lesions from Ethiopian patients [5], as well as in plasma specimens from patients with visceral leishmaniasis [14]. The difference in arginase I expression in plasma and lesions could be due to parasite-related factors or could be a consequence of polymorphisms in host genes, as indicated for other pathologic conditions [23, 24]. Regardless of arginase I source, the data presented here suggest that arginase I levels in plasma are unbalanced in patients with DCL and those with LCL, compared with healthy controls. Furthermore, the relationship between arginase I levels and clinical presentations suggests that arginase I may represent a biomarker for cutaneous leishmaniasis severity.

The arginase I pathway is preferentially driven by the presence of antiinflammatory mediators, such as the lipid mediator PGE_2 [25] and $\text{TGF-}\beta$ [26]. Although PGE_2 production in plasma specimens from patients with DCL and those with LCL did not differ, both patient subgroups exhibited higher values, compared with values for controls, indicating that PGE_2 might be relevant to cutaneous leishmaniasis. PGE_2 can use 4 G-protein-coupled E-prostanoid receptors (prostaglandin E receptors 1–4) [27]. Interestingly, differences between DCL and LCL lesions were only detected in EP2 mRNA levels. PGE_2 signaling through EP2 promotes T-helper type 2 (Th2) immune responses [28] and the suppression of the microbicidal activity of alveolar macrophages through prostaglandin E receptors 2/4,

increasing cAMP levels and inhibiting the assembly and activation of p47phox [29].

Indeed, the potent immunomodulatory effects exhibited by PGE_2 are ambiguous, depending on the profile of in situ mediators. PGE_2 has been shown to synergize with $\text{TNF-}\alpha$ to induce high levels of IL-12 production by dendritic cells [30], whereas $\text{TGF-}\beta$ synergizes with PGE_2 to block $\text{IFN-}\gamma$ and $\text{TNF-}\alpha$ secretion [31]. These effects are associated with suppression of the host immune response and a switch from a Th1 to a Th2 response [32]. Therefore, systemic PGE_2 in LCL, in concert with $\text{TNF-}\alpha$ and IL-12, appears to support effective activation of the immune system, whereas in patients with DCL, PGE_2 and $\text{TGF-}\beta$ have the opposite immunosuppressive effect. Indeed, arginase I plasma levels are upregulated by antiinflammatory mediators and downregulated by proinflammatory mediators, as shown by correlation analysis. This setting appears to be decisive in the clinical outcome of cutaneous leishmaniasis—localized versus metastatic, diffuse disease. The dispersion of mediators found in the plasma suggests that there are subgroups of patients with DCL with possible differences in the pattern of inflammatory response. Since the present study has a limited number of patients with DCL and therefore subgroup analyzes would not have statistical significance, further studies are necessary to clarify this question.

In addition to arginase I, ODC, the subsequent downstream enzyme required for polyamine synthesis, was also enhanced in plasma specimens from patients with DCL, compared with those from patients with LCL. In addition to ODC, L-ornithine

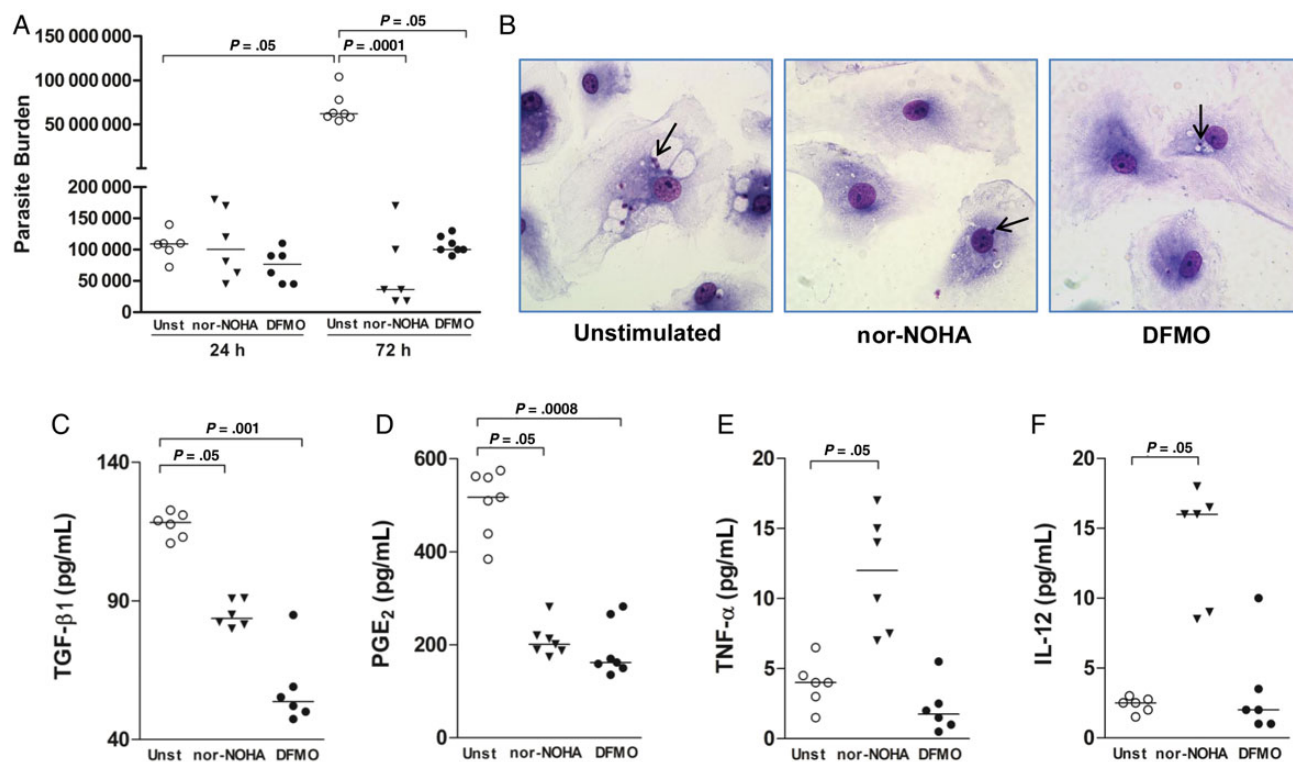


Figure 5. Inhibition of arginase I and ornithine decarboxylase abrogate *Leishmania* replication in infected human macrophages. Infected macrophage monolayers were cultured with medium alone (\circ), NOHA (\blacktriangledown), or DFMO (\bullet) (A). Parasite burden 24 and 72 hours after infection. Each point represents a different donor, and each bar represents the median. B, Micrographs from *Leishmania amazonensis*-infected human macrophages unstimulated or in the presence of 100 mM nor-NOHA or DFMO for 72 hours. Arrows point to amastigotes inside a parasitophorous vacuole. Original magnification $\times 1000$. Bars represent 10 μ m. Differences were evaluated using the Kruskal–Wallis test with the Dunn multiple comparisons post hoc test. Supernatants were collected after 24 hours and assayed for the presence of transforming growth factor β (TGF- β ; C) and prostaglandin E₂ (PGE₂; D), by enzyme-linked immunosorbent assay, and the presence of tumor necrosis factor α (TNF- α ; E) and interleukin 12 (IL-12; F), by the Cytometric Bead Array kit. Statistical significance was determined by a 2-tailed Mann–Whitney test. Data are representative of at least 3 independent assays and were collected in triplicate for each condition. Abbreviation: Unst, unstimulated.

can also be metabolized to proline by ornithine aminotransferase, which is related to collagen production. The increased expression of ODC indicates that the arginase pathway is being deviated toward the first step of polyamine biosynthesis. In addition, both arginase I and ODC exhibit increased expression in DCL lesions, compared with LCL lesions, implying increased polyamine production in situ. Likewise, we observed positive correlations between arginase I mRNA levels and the levels of spermine and spermidine synthase mRNA, the 2 enzymes responsible for polyamine synthesis. Histological analysis of DCL lesions revealed heavily parasitized cells, whereas in LCL lesions, parasites were scarce [13]. Therefore, the high expression of polyamine biosynthetic enzymes may possibly contribute to the intense *Leishmania* proliferation in macrophages in patients with DCL. However, whether the level of the final product polyamine is actually increased in patients with DCL remains unclear and needs to be further investigated.

Arginase I was also related to phospholipase A2, COX-1, COX-2, and prostaglandin E2 synthase, which are all enzymes

involved in PGE₂ syntheses. The relationship between arginase I and the prostaglandin biosynthetic pathway is reinforced by the difference in in situ COX-2 expression between DCL and LCL lesions. COX-2 expression is induced by stimuli such as growth factors and cytokines. TGF- β has been associated with COX-2/PGE₂ expression in peripheral blood lymphocytes [33], where it suppresses the degradation of COX-2 mRNA [34]. In addition, TGF- β enhances arginase activity in macrophages and hence increases polyamine release [35]. In agreement with the literature, our data demonstrate that both TGF- β and COX-2 are increased in patients with DCL and are positively correlated with arginase I, suggesting their involvement in its production.

The inhibition of polyamine pathway enzymes is efficient for experimental *Toxoplasma* and trypanosome infections [36] and has been used as a treatment in patients with African sleeping sickness [37]. Arginase and ODC inhibition directly affects the production of polyamines, which are critical for the survival and replication of *Leishmania* parasites [6, 38]. Hence, in our model, control of *L. amazonensis* replication in macrophages

treated with nor-NOHA (arginase inhibitor) and DFMO (ODC inhibitor) could be mediated by a decrease in polyamine levels. Furthermore, nor-NOHA and DFMO appear to modulate the inflammatory response. Arginase and ODC inhibition decreased levels of TGF- β and PGE₂, which are crucial to parasite survival and proliferation [39]. Moreover, treatment with nor-NOHA increased levels of TNF- α and IL-12, which can activate macrophages to produce nitric oxide [40], a potent leishmanicidal molecule. Accordingly, treatment with the arginase inhibitor nor-NOHA altered the cytokine profile, suggesting that the functional status of macrophages was changed from classical activation to alternative activation. Interesting, DFMO treatment did not increase proinflammatory cytokine production, although DFMO treatment was as efficient as nor-NOHA in controlling parasite load. These data suggest that polyamines are sufficient to support *L. amazonensis* survival and multiplication and that the inhibition of polyamines is decisive for the outcome of infection.

Taken together, our data implicate the local and systemic release of arginase I, prostaglandins, and polyamines in the inability of patients with DCL to mount an efficient immune response against *L. amazonensis* infection, providing a favorable environment for parasite replication and the dissemination of the disease. Considering that DCL is still without an effective treatment, the discovery of novel targets for chemotherapy is extremely important. Our study highlights arginase I, ODC, and COX-2 as promising targets for DCL treatment, and inhibitors of these enzymes are already used successfully in the control of other pathologic conditions. Drugs interfering with these metabolic pathways are commercially available and could be used in future clinical trials, reestablishing a balanced inflammatory response to control parasite replication in DCL.

Notes

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